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Efficacy of Immunization of High-Risk Infants Against Hepatitis B Virus Evaluated by Polymerase Chain Reaction

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The polymerase chain reaction (PCR) is a rapid and very sensitive method to detect viral genomes. In the present study, the efficacy of immunization against hepatitis B virus (HBV) of high-risk infants was evaluated by PCR. Twenty-nine infants born to 24 HBeAg-positive carrier mothers were given hepatitis B immune globulin (HBIG) at birth and thereafter received repeated inoculations of plasma-derived vaccine or HBIG, or both, within 1 year. Serum samples at 1 year following immunization were stored at −40°C for later analysis using PCR to detect HBV-DNA. When HBV genomes were detected in infants, the DNA sequences in the S gene of HBV were determined. Of 29 infants, 2 were positive for HBV-DNA at the 1 year following immunization; one had the HBV containing only the wild-type sequence in the S gene and became negative for HBV-DNA during the follow-up period. In contrast, another had the HBV, which contained nucleotide substitutions that altered the expression of the common group-specific determinant ''a'' of the S gene and resulted in clinical hepatitis with viral persistence. PCR analysis suggests that immunization against HBV prevents effectively high-risk infants from mother-to-child transmission. Even then, however, it is possible that amino acid substitutions in the ''a'' determinant of the S gene are associated with failure of conventional immunization against HBV. ***J. Med. Virol. 53:255–260, 1997.***

KEY WORDS: hepatitis B vaccine; HBV-DNA; PCR

INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide. Although its prevalence shows striking geographical and ethnic variations [Zaman et al., 1985; Beasley, 1988; Koff, 1993], the prevalence of HBV carriers is high in Africa and east Asia, ranging from 5% to 20%; intermediate in South America, ranging from 2% to 7%; and low in North America and western Europe, ranging from less than 0.5%. Persistent HBV infection evolves into chronic hepatitis and a proportion of chronic cases progress to liver cirrhosis, hepatocellular carcinoma or both [Koff, 1993; Kato et al., 1994]. Previous epidemiological studies demonstrated a strong association between persistent HBV infection and hepatocellular carcinoma, especially in several areas in Asia and Africa [Zaman et al., 1985; Beasley, 1988]. In Japan, the HBV prevalence rate has decreased during the past 20 years [Matsuo et al., 1990]. This resulted mainly from the reduction of horizontal transmission of HBV in infancy, but mother-to-child transmission of HBV occurred steadily without immunization.

Immunization of infants against HBV is of particular importance because perinatal transmission of HBV is the most common mode of infection in some regions of the world [Beasley et al., 1983; Wong et al., 1984; Stevens et al., 1987]. In addition, since therapy for hepatitis B is effective only in a proportion of patients, prevention of infection by immunization is a key strategy to eradicate the HBV-associated liver disease. Since the start of passive and active immunization against HBV of high-risk infants, several groups have documented the effectiveness of immunization, where the efficacy of immunization was evaluated by serological virus markers only such as HBsAg and antibody to HBsAg (anti-HBs) [Eto and Shiraki, 1989; Marion et al., 1994; Margolis et al., 1995; Whittle et al., 1995]. However, some evidence indicates that the HBV genome can be detected in sera by using the polymerase

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TABLE I. Characteristics of Infants Studied

Variable	Infants (n = 29)	Mothers (n = 24)
Sex (M/F)	15/14	
HBeAg (+/−)		24/0
Mean age at delivery		29
Range		(19–39)

chain reaction (PCR) from HBsAg-negative patients or even patients with anti-HBs and chronic liver disease [Kuhns et al., 1992; Zhang et al., 1993; Hou et al., 1995; Kato et al., 1996]. In the present study, the efficacy of immunization against HBV of infants born to both HBsAg- and HBeAg-positive mothers was evaluated by PCR to detect HBV-DNA. In addition, to elucidate a possible mechanism of perinatal transmission of HBV in two infants who were positive for HBV-DNA at 1 year following immunization, DNA sequences in the S gene of HBV of two infants and their mothers were analyzed.

MATERIALS AND METHODS

Twenty-nine infants born to 24 mothers with both HBsAg and HBeAg between 1984 and 1993 were studied (Table I). The sample population included 15 men and 14 women. All infants received 1 ml of hepatitis B immune globulin (HBIG) intramuscularly at birth and thereafter received repeated infections of 10 μg of plasma-derived vaccine two or more times together with or without HBIG within 1 year. The infants were followed for more than 1 year following immunization. Serum samples were obtained during the follow-up period and stored at −40°C for later analysis of HBV-DNA by PCR. Tests for HBsAg and anti-HBs were carried out using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Behring Diagnostics GmbH, Frankfurt, Germany) and a passive hemoagglutination assay kit (Eisai, Tokyo, Japan), respectively.

DNA was extracted from each serum sample as described previously [Hamasaki et al., 1994; Inokuchi et al., 1996]. The extracted DNA was subjected to analysis using a nested PCR to detect HBV genome. The oligonucleotide primers used in the present study are presented in Table II. Briefly, the extracted DNA was amplified by the first stage of PCR, using the set of outer primers for 40 cycles. The reaction cycle involved denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min. The second stage of PCR was performed for 40 cycles using the set of inner primers. The reaction condition involved denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and primer extension at 72°C for 1 min. The PCR products were extracted with phenol-chloroform, precipitated with ethanol and later separated by electrophoresis on a 2% agarose gel. The separated band corresponding to the HBV-DNA fragment was visualized using ethidium bromide staining. In the serum sample that was positive for HBV-DNA by PCR, DNA sequences in the S gene of HBV were determined. Briefly, the PCR product was fractionated on a 1.2% agarose gel, and the band visualized with ethidium bromide staining was removed. The HBV-DNA fragment purified with a QIAEX gel extraction kit (Qiagen, Chatsworth, CA) was inserted into pGEM-T plasmid (Promega, Madison, WI) and cloned. Each clone was sequenced using a 377-DNA sequencing system and a cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequence data were analyzed by 377 sequencing analysis software version 2.1.1 (Applied Biosystems, Foster City, CA).

TABLE II. Oligonucleotide Sequences of Primers for HBV-DNA

Primers	Sequence
Outer primers	
Sense	5′-TACAGGCGGGGTTTTTCTTG-3′
Antisense	5′-AAGCCCTACGAACCACTGAA-3′
Inner primers	
Sense	5′-TCTGCGGCGTTTTATCATAT-3′
Antisense	5′-AAGCCCTACGAACCACTGAA-3′

RESULTS

Detection of HBV-DNA by PCR at the 1 Year Following Immunization

The 29 infants were given HBIG at birth and thereafter received injections of plasma-derived vaccine two or more times together with or without HBIG within 1 year (Table III). All 29 infants were negative for HBsAg, and all 27 infants tested were positive for anti-HBs at the 1-year following immunization. In addition, the follow-up study indicated that 28 of 29 infants were negative for HBsAg and positive for anti-HBs at the 2- to 4-year following immunization. However, when the serum samples at 1 year following immunization were subjected to analysis of HBV genome by a nested PCR, irrespective of the serological virus markers, HBV-DNA was detected in two infants, although both were positive for anti-HBs at that time.

Clinical Course in Two Infants Who Were Positive for HBV-DNA at 1 Year Following Immunization

The two infants in whom HBV-DNA was detected by PCR at 1 year following immunization were followed closely for more than 3 years. In one infant (case 28), the levels of serum alanine aminotransferase (ALT) were normal throughout the study, and HBV-DNA could not be detected by a nested PCR at 3 years following immunization (Fig. 1A). In contrast, another infant (case 29) had a flareup of hepatitis manifested by the elevated levels of serum ALT 14 months after birth (Fig. 1B). In this case, HBV-DNA was detected at 6 years following immunization, while anti-HBs converted to HBsAg during the follow-up period.

Analysis of DNA Sequences in the S Gene

Since the efficacy of immunization with HBIG and vaccine could be influenced by mutations within the S

TABLE III. Virological and Serological Data in 29 Infants Following Immunization

Case No.	Sex	No. of additional immunization		HBsAg/anti-HBs status		HBV-DNA at 1-yr
		Vaccine	HBIG	at 1 yr	at 2–4 yr	First PCR/second PCR
1	M	4	1	−/+	−/+	−/−
2	M	4	1	−/+	−/+	−/−
3	F	3	2	−/+	−/+	−/−
4	F	3	1	−/+	−/+	−/−
5	M	3	1	−/+	−/+	−/−
6	F	3	1	−/+	−/+	−/−
7	F	3	1	−/+	−/+	−/−
8	F	3	1	−/+	−/+	−/−
9	F	3	1	−/+	−/+	−/−
10	M	3	1	−/+	−/+	−/−
11	M	3	1	−/+	−/+	−/−
12	F	3	1	−/+	−/+	−/−
13	M	3	1	−/+	−/+	−/−
14	F	3	1	−/+	−/+	−/−
15	M	3	1	−/+	−/+	−/−
16	F	3	1	−/+	−/+	−/−
17	F	3	1	−/+	−/+	−/−
18	M	3	1	−/+	−/+	−/−
19	F	3	1	−/+	−/+	−/−
20	M	3	1	−/+	−/+	−/−
21	F	3	1	−/+	−/+	−/−
22	F	2	3	−/+	−/+	−/−
23	M	2	3	−/+	−/+	−/−
24	M	2	3	−/+	−/+	−/−
25	M	2	2	−/+	−/+	−/−
26	M	2	2	−/NT	−/+	−/−
27	F	2	1	−/NT	−/+	−/−
28	M	2	2	−/+	−/+	−/+
29	M	2	2	−/+	+/−	+/+

NT, not tested.

gene of HBV [Carman et al., 1990; Fujii et al., 1992; Okamoto et al., 1992; Hino et al., 1995], DNA sequences in the S gene of HBV were determined in two infants who were positive for HBV-DNA at 1 year following immunization and in their mothers. In one infant (case 28) whose HBV-DNA was detected by a nested PCR at 1 year but became undetectable at 3 years following immunization (Fig. 2A), all five clones derived from the serum sample at 1 year following immunization showed the wild-type sequence of HBV/adr the same as the sequence found in the mother's serum. In another infant (case 29) who suffered from hepatitis with viral persistence during the follow-up period, the serum samples at 2 months, 1 year, and 6 years following immunization were available for analysis of DNA sequences in the S gene of HBV. (Fig. 2B). The infant had a mixed viral population of the wild-type HBV and the mutant-type HBV containing point mutations, C-to-A at nucleotide 513 and G-to-A at nucleotide 587, resulting in amino acid substitutions from proline to glutamine at the 120th codon and from glycine to arginine at the 145th codon of the S gene, respectively, at 2 months following immunization. Moreover, the follow-up study indicated that the infant had only the mutant-type HBV identical to that found at 2 months, although other minor amino acid substitutions in the sequence of the S gene were also found at the 6-year following immunization. To determine whether the

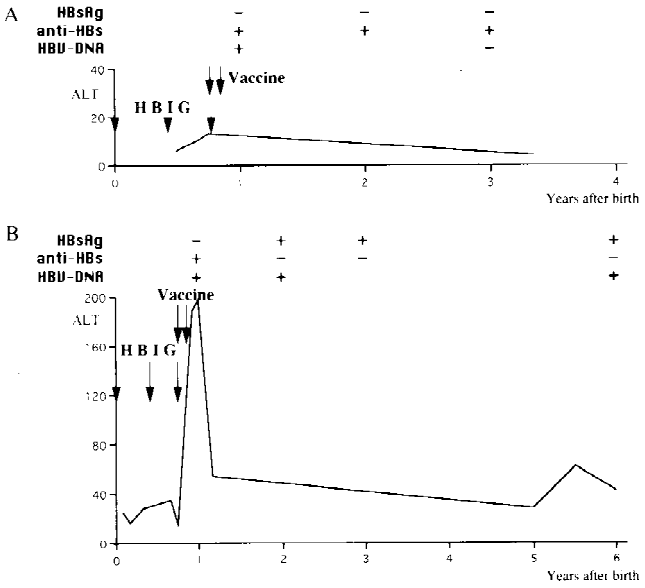

Fig. 1. Clinical course in two infants who were positive for HBV-DNA by PCR at 1 year following immunization. The two infants (cases 28 and 29) who were positive for HBV-DNA by PCR at 1 year following immunization were followed closely in our hospital. **A:** Clinical course of case 28, in which HBV-DNA could not be detected at 3 years following immunization. **B:** Clinical course of case 29 in which HBV-DNA was persistently detected despite immunization.

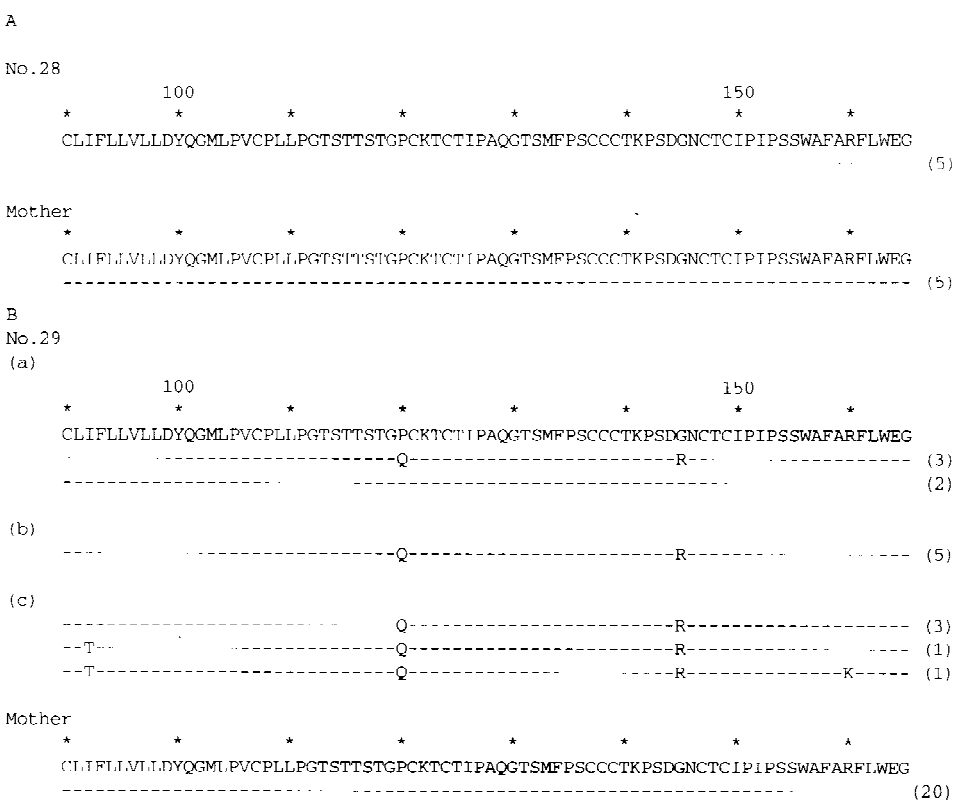

Fig. 2. Amino acid sequences covering "a" determinant of the S gene codon in two infants and in their mothers. The amino acid sequence in the wild-type S gene (HBV/adr) is shown in the upper column in each case. The number of clones analyzed in each case is shown in parentheses. **A:** Amino acid sequences of the S region in case 28 and his mother. **B:** Amino acid sequences of the S region in case 29 at 2 months (a), at 1 year (b), and at 6 years (c) following immunization, and in his mother.

mutant-type HBV was transmitted from the mother to the infant, the mother's serum sample was studied. Although 20 clones were established from the mother's serum sample and subjected to analysis of HBV-DNA sequences in the S gene, all clones contained the wild-type sequence of HBV in the S gene.

DISCUSSION

The efficacy of immunization against HBV of infants was evaluated by analysis using PCR to detect HBV-DNA, because PCR can detect HBV-DNA in amounts as low as 10^{-5} pg of HBV-DNA [Kaneko et al., 1989; Sumazaki et al., 1989] and because HBV genomes are often detected in patients with chronic liver disease who are negative for HBsAg or positive for anti-HBs [Kuhns et al., 1992; Zhang et al., 1993; Hou et al., 1995; Kamito et al., 1996]. Based on the fact that infection by HBeAg-positive carrier mothers is most likely to result in a chronic carrier state in infected infants [Beasley et al., 1983; Wong et al., 1984; Stevens et al., 1987], possibly by induction of immunological tolerance to HBeAg in utero [Milich et al., 1990], 29 infants who were born to both HBsAg- and HBeAg-positive mothers and received immunization with HBIG and vaccine were studied. All the 29 infants were negative for HBsAg, and 27 infants were positive for anti-HBs at 1 year following immunization. However, by analysis using the same serum samples with a nested PCR, HBV-DNA was identified in 2 of 29 infants. During the follow-up period, the HBV genome became undetectable by nested PCR in one infant (case 28) who had only the wild type of HBV in the S gene. In contrast, another infant (case 29) who had the mutant-type of HBV containing amino acid substitutions within "a" determinant and immediately upstream of this determinant in the S gene became a HBV carrier with clinical manifestations of hepatitis. The time course analysis in this infant indicated that the wild-type HBV was replaced with the mutant-type HBV during immunization. Thus, the result using PCR, ensures the effectiveness of immunization against HBV to prevent high-risk infants from infection.

The HBV variants that can escape from immunization with HBIG and vaccine are an inevitable problem of the conventional immunization against HBV. Since the "a" determinant of the S gene of HBV is a main target for conventional immunization [Carman et al., 1990], amino acid substitutions within "a" determinant of the S gene are responsible for vaccine-induced escape mutants, among which a substitution of arginine for glycine at 145th codon of the S gene as was identified in the present study is most common [Carman et al., 1990; Fujii et al., 1992; Okamoto et al., 1992; Zuckerman et al., 1994; Oon et al., 1995]. In addition, cur-

rent studies showed that amino acid substitutions or insertions immediately upstream of this determinant in the S gene are also associated with breakthrough of HBV variants [Karthigesu et al., 1994; Yamamoto et al., 1994; Carman et al., 1995; Hou et al., 1995; Oon et al., 1996]. A substitution of glutamine for proline at position 120 of the S gene found in our case of viral persistence would contribute to escape from the conventional immunization against HBV. The analysis of DNA sequences in the S gene of HBV showed that the mother of the infant who resulted in viral persistence despite immunization had only the wild-type sequence. Okamoto et al. (1992) reported two cases of maternal transmission of HBV in infants immunized with HBIG and vaccine. In one case, the carrier infant and her mother had similar HBV variants. However, in another case, the DNA clones from the carrier infant had a mutation of "a" determinant in the S gene, but those from her mother had no such mutations, as seen in this study. It is therefore possible that a very minor proportion of the HBV variants is transmitted from a carrier mother to a child and selected in infancy during conventional immunization against HBV.

Since the pre-S2 and pre-S1 regions of HBV harbors T-lymphocyte epitopes that are much more potent than the S region [Milich et al., 1985a], pre S2-containing vaccines can induce the antibody production more effectively through the helper T-cell response than the conventional vaccines that lack the pre-S domains [Milich et al., 1985b; Itoh et al., 1986; Neurath et al., 1986]. In fact, a group of infants receiving the pre-S2-containing vaccine did produce higher titers of anti-HBs than were produced by a group of infants given plasma-derived vaccine [Soulie et al., 1991]. Moreover, Noto et al. (1997) reported that an infant infected with a vaccine-escape variant of HBV was immunized successfully with the pre-S2-containing vaccine, resulting in the complete eradication of the variant. Although conventional immunization against HBV of high-risk infants is effective even by using PCR, the pre-S2-containing vaccine should be considered in cases of the HBV variants with the altered expression of the "a" determinant of the S gene.

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